A case of Paroxysmal Nocturnal Hemoglobinuria caused by a germline mutation and a somatic mutation in PIGT

Running title:
PNH caused by PIGT mutations

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Key Point: A carrier of a deleterious splice site mutation in \textit{PIGT} acquired a second hit in \textit{PIGT} and developed PNH.

Abstract

To ascertain the genetic basis of a PNH case without somatic mutations in \textit{PIGA}, we performed deep next-generation sequencing on all exons of known genes of the GPI-anchor synthesis pathway. We identified a heterozygous germline splice site mutation in \textit{PIGT} and a somatic 8MB deletion in granulocytes affecting the other copy of \textit{PIGT}. \textit{PIGA} is essential for GPI-anchor synthesis whereas \textit{PIGT} is essential for attachment of preassembled GPI-anchor to proteins. While a single mutation event in the X-chromosomal gene \textit{PIGA} is known to cause GPI-anchored protein deficiency, two such hits are required in the autosomal gene \textit{PIGT}. Our data indicate that PNH can occur even in the presence of fully assembled GPI if its transfer to proteins is defective in hematopoietic stem cells.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia that results from the expansion of hematopoietic stem cells (HSC) that are deficient for glycosylphosphatidylinositol (GPI), a glycolipid moiety that anchors more than a hundred of different proteins to the cell surface\textsuperscript{13}. PNH patients were reported to be deficient for an initial step in the GPI-anchor synthesis that is catalyzed by the GPI-GlcNAc transferase\textsuperscript{3,6,7} and somatic mutations in the X-chromosomal gene \textit{PIGA} that encodes a subunit of this transferase complex\textsuperscript{8}, are regarded as the causative event in the predominant number of PNH cases\textsuperscript{2-5,7,9}. However, in a small number of PNH cases with a clear GPI-anchor deficiency no mutations in \textit{PIGA} have been found.

In this work we report about two mutation events, a germline splice site mutation as well as a somatic deletion in \textit{PIGT}, which is another gene of the GPI-anchor synthesis pathway, that we identified performing next-generation sequencing in a PNH patient with wild-type \textit{PIGA}.

Methods

Patient sample

This study was conducted in accordance with the Declaration of Helsinki. Genetic analysis was performed after approval by ethical committee and informed consent.

Targeted genomic sequencing, arrayCGH and FISH

For the targeted enrichment of exons of all known GPI-anchor synthesis genes (Table S1) we used a customized SureSelect library (Agilent) as previously described\textsuperscript{10}. Genomic DNA of the patient and nine controls was isolated from whole blood and enriched for GPI pathway exons according to the manufacturer’s protocol, followed by single-read cluster generation on a Cluster Station (Illumina). The captured, purified, and clonally amplified library was then sequenced on an Illumina Genome Analyzer IIx and mapped to the human reference sequence GRCh37, resulting in a mean coverage of above 300 fold for all exons and more than a 10 fold coverage for more than 95% of the target region. Variants were detected with SAMtools\textsuperscript{11}, annotated with ANNOVAR\textsuperscript{12} and further analyzed in GeneTalk\textsuperscript{13}.

For the detection of exon deletions we first counted the reads per exon and normalized this value for each sample by the total number of reads that were mapped to the target region. This normalized read count per exon was used to compute the mean and variance for the coverage per exon in all analyzed samples. Exons with a normalized coverage that was two standard deviations below the mean were classified as partially deleted in a subpopulation of cells and further analyzed.
Array comparative genomic hybridization (arrayCGH) was carried out on genomic DNA isolated from a peripheral blood draw using whole-genome 1M Oligonucleotide-Array (Agilent) to confirm the deletion of PIGT and to characterize its extend. Analysis was performed with Feature Extraction and CGH Analytics software (Agilent) as described previously. The CNV involving PIGT was further analyzed with FISH using BAC clone RP3-337O18 in metaphases of PHA stimulated T-lymphocytes and granulocytes that were enriched by a Ficoll gradient.

**Cell culture and FACS**
We cloned a coding region of human PIGT (NM_015937) from a cDNA library derived from placenta, tagged with FLAG at the N-terminus and subcloned it into pME. PIGT mutant with skipped exon 11 was generated by site-directed mutagenesis. Mutant and wild-type PIGT plasmids were transfected into electroporation into PIGT-deficient Chinese Hamster Ovary (CHO) cells expressing human GPI-anchored proteins, CD55 and CD59 as previously described. Two days later, lysates were applied to SDS-PAGE and western blotting against anti-FLAG antibody to determine levels of expressed PIGT. The levels of CD55 and CD59 restored at the cell surface were determined by FACS.

**Results and discussion**
We performed targeted enrichment of all exons of genes involved in GPI-anchor synthesis followed by ultra-deep sequencing in a female patient with classical hemolytic PNH that is negative for mutations in PIGA. The patient was diagnosed with hemolytic anemia with a negative direct antiglobulin test (DAT) at the age of 44 years and experienced frequent hemolytic crises, abdominal pain, diarrhea, headache, arthralgia, dyspnea and fatigue in the following years. At the age of 49 years a flow cytometric analysis was performed that showed reduced expression of GPI-anchored proteins on blood cells (Figure 1A). DNA was isolated from blood at that time and subjected to ultra-deep sequencing. The patient was started on eculizumab due to PNH-related symptoms, soon after it became available six years ago and responded to this treatment (see supplemental data for a detailed clinical description of the patient).

We detected a significant reduction in the coverage of all PIGT exons in the DNA extracted from blood compared to other genes of the GPI-anchor synthesis pathway, which suggested a deletion of this gene in a subpopulation of cells (Figure 2A). We performed array CGH to measure the full extent of the CNV and detected a 8MB deletion, arr20q11.23q13.12 (Figure 2A). In order to clarify which subpopulation was affected by the deletion we used a FISH probe (RP3-337O18) targeting the CNV interval in T-lymphocytes and granulocytes. While we did not observe any deletion in full metaphases of T-lymphocytes, 92% of the evaluated granulocyte interphase nuclei showed only a single signal of for RP3-337O18 suggesting a heterozygous deletion including PIGT in a myeloid stem cell that occurred as a somatic event (Figure 2B).

The mutation analysis of the deep sequencing data revealed a single nucleotide substitution in PIGT affecting the splice acceptor site of intron 10, NM_015937:c.1401-2A>G (Figure 2C). From 1463 sequence reads that cover the splice site 1239 showed the base substitution, suggesting that the mutation is present on the chromosome without the somatic deletion involving PIGT. We also measured the splice site mutation in a heterozygous state in ABI Sanger sequences of DNA that was extracted from epithelial cells of a buccal swab providing further evidence that c.1401-2A is the germline event (Figure 2D). Based on these findings we hypothesized that the somatic deletion of the wildtype allele of PIGT occurred in a myeloid stem cell and resulted in a clone that is hemizygous for PIGT. In this clone the single remaining copy of PIGT is functionally impaired due to the splice site mutation that results in skipping of 84bp of exon 11 deleting 28 highly conserved amino acids in PIGT.
We analyzed the functional effect of the germline splice site mutation in *PIGT*-null CHO cells. While the transfection of wild-type *PIGT* into these cells restored the levels of wild-type GPI-linked proteins CD55 and CD59 at the cell surface, the transfection of the mutant only leads to a minor increase of CD55 surface expression but almost no CD59 expression at comparable *PIGT* protein levels (Figure 1B).

In contrast to the X-chromosomal *PIGA*, all other known genes involved in the GPI-anchor synthesis pathway, including *PIGT*, are found on autosomes and inactivating mutations in these genes have to occur on both alleles in the same cell to result in a GPI-anchor deficiency. The co-occurrence of two mutations in the same gene is a situation that is similar to hereditary cases of retinoblastoma that have been explained by a two hit model of one inherited mutation and one somatic mutation in *RB1*. Therefore, individuals that are heterozygous for mutations in autosomal genes that impair the GPI-anchor synthesis, such as the reported splice site mutation in *PIGT*, might have an increased risk to develop PNH.

While PIGA catalyzes the first step of the GPI-anchor synthesis, *PIGT* is a component of the transamidase complex that is required for attachment of preassembled GPI to proteins. Therefore, even in the presence of fully assembled GPI anchors PNH can occur. This suggests that not only the specific defect in the GPI anchor synthesis that is caused by *PIGA* mutations, but also a GPI-anchored protein deficiency that is due to mutations in other genes of the pathway may predispose for PNH. Interestingly, a deletion on 20q is also a recurrent somatic abnormality in myelodysplastic syndrome, however, at the current moment it is not clear whether the loss of heterozygosity of other genes in this region contributes besides *PIGT*, contributes to the clonal expansion.

Recent findings of congenital GPI-deficiencies also shed a new light on the clinical feature of hemoglobinuria. While no hemolysis was reported for patients with germline mutations in *PIGN*, *PIGM*, *PIGO*, *PIGL*, *PIGV* and even *PIGA* and *PIGT*, chronic hemolysis was described in patients with a congenital CD59 deficiency that responds to eculizumab therapy. Further studies are therefore required to elucidate how mutations in GPI pathway genes contribute to the different phenotypic features and to which extent additional somatic events occur.

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**Author Contributions**

Contribution: P.M.K., D.P performed research, analyzed the data. U.K., J.H., C.S. performed sequencing studies, B.H., H.S., provided patient samples and characterized the patient, A.H. provided patient samples, performed research and analyzed data, E.K. performed arrayCGH, B.T. performed the FISH analysis, Y.M., performed cell culture experiments, H.S., B.H., P.M.K., designed the study. E.K. H.N., P.N.R., Y.M., J.H., T.K., S.M. wrote the paper.

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References


Figure 1: A) Expression of GPI-anchored proteins on patient’s peripheral blood cells and reduced activity of PIGT mutant in restoring surface expression of GPI-anchored proteins after transfection into PIGT-null cell lines. A) Expression of CD58 and CD59 (A, left) on red cells, CD24 and CD66b on neutrophil granulocytes (A, middle) and CD48 on B and T lymphocytes (A, right). The first row shows the expression of GPI-AP at the time of ultradeep sequencing (4.5 years after start of eculizumab), the second row shows expression of GPI-AP in a healthy control. In healthy controls CD58 and CD59 are expressed on >99.9% and >99.5 of red cells, CD24/CD66b is expressed on >99.8% of neutrophil granulocytes (A, second row). In contrast, the patient shows a mosaic of cells with normal expression of GPI-anchored proteins and cells with reduced or completely missing expression of GPI-AP on erythrocytes (left hand panels) or neutrophil granulocytes (middle panel). The cell populations which completely lack expression of the respective GPI-AP are indicated by arrows, the populations with reduced GPI-AP expression are marked by asterisks. The patient did not receive any blood transfusions over a period of three months prior to this measurement. Expression of the GPI-AP CD48 on T-lymphocytes was normal, whereas a subpopulation of B-lymphocytes did not express the GPI-AP CD48. The percentages of cells with reduced or absent GPI-AP, i.e. PNH cells and normal range is shown in the supplementary data. B) PIGT deficient CHO cells were transiently transfected with wild type or a mutant version skipping exon 11 of transcript NM_0015937. (B, left) Restoration of the cell surface protein levels of wild-type PIGT and the mutant PIGT lacking 28 amino acids encoded by exon 11 was assessed by flow cytometry. Wild-type PIGT efficiently restored expression levels of CD59 and CD55 at the cell surface (dotted black lines), whereas the mutant PIGT did not rescue CD59 and only partially rescued CD55 expression (solid black lines). Dark shades, empty vector; light shades, isotype-matched control. (B, right) Expression levels of transfected wild-type and the mutant HA-tagged PIGT. PIGT proteins were determined by western blotting with anti-HA, GAPDH, loading control. Normalized PIGT levels are shown at the bottom.
Figure 2: Ultra-deep sequencing of all exons of genes involved in GPI-anchor synthesis reveals two mutation events in PIGT, a germline splice site mutation and a somatic deletion. (A) DNA was isolated from whole blood and enriched for all exons of genes involved in GPI-anchor synthesis and subjected to ultra-deep sequencing. The coverage of PIGT exons was significantly reduced compared to exons of all other GPI-anchor synthesis genes, suggesting a deletion involving PIGT. The extent of the deletion was further characterized by arrayCGH comprising in total 8MB, arr20q11.23q13.12. (B) Fluorescence in situ hybridization with BAC clone RP3-337O18 (G) and a probe targeting the centromere of chromosome 20 (R) was used to analyze the deletion in T-lymphocytes and granulocytes. While two signals of RP3-337O18 were present in all complete metaphases of T-lymphocytes, the majority of granulocytes showed only one signal for RP3-337O18, indicating a somatic deletion in a myeloid lineage. (C) A single nucleotide substitution in PIGT affecting the splice acceptor site of intron 10, NM_015937:c.1401-2A>G, was observed in the ultra-deep sequencing data of DNA extracted from whole blood. In total, 1463 sequence reads covered the canonical splice site and 85% of these reads showed the alternate base, indicating that the mutation is present on the undeleted haplotype of PIGT. (D) The splice site mutation was validated by ABI Sanger sequencing and shown to be heterozygous in DNA extracted from epithelial cells of a buccal swap, confirming its presence in different tissues.
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